

REMARKS/ARGUMENTS

In response to the Office Action of August 30, 2005, Applicants have amended the claims, which, when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested.

The Examiner has acknowledged Applicants' election of claims in the response to the restriction requirement filed on May 10, 2005. Therefore, group II, claims 14-16 and 18 have been withdrawn from consideration by the Examiner. As presently amended, claim 14 recites a process for purifying on a large scale a product from a feedstock wherein the product is a rapamycin or a derivative thereof or an ascomycin or derivative thereof. Cyclosporin, which is not part of the elected subject matter, is no longer recited by this claim. Applicants therefore respectfully request that the Examiner reinstate claim 14. In addition, since claims 15 and 16 depend from presently amended claim 14, Applicants respectfully request that the Examiner also reinstate these two claims.

In the Office Action, the Examiner has stated that the Information Disclosure Statement (IDS) filed on 9/16/03 fails to comply with 37 CFR 1.98 (a)(1). The Examiner has therefore placed the IDS in the application but information referred to therein has not been considered. It is respectfully submitted that the IDS previously submitted contained all of the necessary information to identify the references cited by the Applicants so as to allow the Examiner to consider the art. Moreover, as of the submission date of the IDS, **September 16, 2003**, there was no requirement under 37 C.F.R. 1.98(a)(1) for "a column that provides a blank space next to each document to be considered, for the examiner's initials. *See* 37 C.F.R. §1.98 "[...]" paras. (a) and (c) revised and par. (e) removed, 69

FR56481, Sept. 21, 2004, **effective Oct. 21, 2004.**] Thus, as of September 16, 2003, the submission date of the IDS in this application, the IDS was in compliance with 37 C.F.R. §1.98 in effect at the time.

Even if there was a requirement in place at the time for a blank space next to each document to be considered for the examiner's initials, the IDS contained ample space for the Examiner to initial each reference after the art was considered. Nonetheless, in order to expedite prosecution of the present application, a newly prepared IDS containing the same information as previously filed has been submitted herewith. It is not believed that any additional fee is required, but if an additional fee is required, please charge the same to Deposit Acct No. 04-1121.

The Examiner has objected to Claims 21 and 22 as allegedly substantially duplicative of claims 19 and 20. Claims 19-22 are presently canceled from the application. The objection to claims 21 and 22 is therefore moot.

Claims 11-13, 19 and 21 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. In particular, the position of the Examiner is that the phrase "a rapamycin or an ascomycin" is indefinite because use of the articles "a" and "an" make it appear that Applicants are intending to claim two genera of compounds. According to the Examiner however, it is unclear what members belong to the two genera. The Examiner has inquired how the genera differ from the compounds (i.e., a rapamycin and an ascomycin). For example, the Examiner has stated on page 4, paragraph 8 of the Office Action that the fourth paragraph on page 3 of Applicants' specification "merely lists these five compounds but does not describe the families." The

Examiner has also inquired how FK506 can be an ascomycin, noting that FK506 has a trans double bond in the macrolactone skeleton while ascomycin has a cis double bond at the same position.

As presently amended, claim 11 recites “or a derivative thereof” after each of the terms “rapamycin” and “ascomycin”. The articles “a” and “an” as they appeared before “rapamycin” and “ascomycin” have been canceled from the claim. Support for these amendments may be found throughout the specification, e.g., page 3, lines 18-24. In addition, Applicants submit herewith as Exhibit A, Uchida, T. et al. (2002) “Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation” *Internat. J. Mol. Med.* 9:141-145” that describes the relationship of the compounds recited in the presently amended claims. As described on page 141 of the paper, Ascomycin is a C-21 ethyl analog of FK506 and is a 23-member macrolide. Ascomycin is also known as immunomycin and FK520. *See* Figure 1. As may be seen from the dates of the references cited on page 141 of the paper, the relationship among ascomycin and derivatives thereof, and rapamycin and derivatives thereof, was known in the art at the time the present application was first filed. In view of the amendments to the claims and the foregoing remarks, withdrawal of the rejection of claims 11-13, 19, and 21 under 35 U.S.C. § 112, second paragraph is respectfully requested.

Claims 17, 20 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, because the limitation “FK506” is recited in the last line of each of these claims. It is the Examiner’s position that no antecedent basis exists for this term. As stated above, Claim 11 has been amended to include the phrase “or a derivative therefore” after each of the terms “rapamycin” and “ascomycin.” Since FK506 is a

derivative of ascomycin (*see* specification, page 3, lines 23-24), claim 11 as presently amended provides the necessary antecedent basis for this term with respect to claim 17. Claims 20 and 22 have been canceled from the application, and the rejection of these claims is therefore moot. In view of the foregoing, it is respectfully requested that the rejection of Claim 17 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

Claims 21 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for ultimately depending from non-elected claim 14. Claims 21 and 22 are presently canceled from the application. The rejection is therefore moot.

Claims 11, 19 and 21 have been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by U.S. Patent 5,359,060 to Hauske.

Hauske has been cited for teaching purification of FK520 by counter current distribution using 10:1 heptane:acetonitrile solvent system. In making the rejection, the Examiner has directed Applicants' attention to column 9, lines 54-59 and column 10, lines 40-43.

Column 9, lines 54-62, provides:

The concentrate was subjected to four tube counter current distribution in 20 liter carbuoys using 10 liter top layer and 1 litter bottom layer per carbuoy of a heptane/acetonitrile 10/1 system. The active bottom layers were collected, combined and concentrated. The material was further purified via filtration through Florisil (washing with hexane, hexane/methylene chloride and methylene chloride, successively, with a gradual increase in methylene chloride).

It is noted that in making the rejection, the Examiner included Claim 11 as part of the set of claims being rejected, yet concluded that only Claims 19 and 21 were taught.

For the sake of completeness, this response assumes that the Examiner's conclusion reached in this rejection also pertains to Claim 11.

In contrast to Hauske, claim 11 of the present application recites that heptane and acetone or heptane and isopropanol is used in the lighter phase of the counter current separation and water and acetone or water and isopropanol is used for the heavier phase. Nowhere in Hauske is there a teaching for the use of two different solvent mixtures for the two different phases, wherein the lighter phase flows counter to the heavier phase. Moreover, nowhere in the reference is the mixture of these solvents even mentioned. Claim 11 is therefore distinguished from the teaching provided by Hauske. Withdrawal of the rejection of claim 11 under 35 U.S.C. § 102 (b) is therefore warranted. Claim 19 and 21 have been canceled from the application and the rejection as pertains to these two claims is therefore moot.

Claims 19-22 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by EP 652,219 A1 (hereinafter "Gletsos"). Gletsos teaches the purification of both Rapamycin and FK506 by extraction. In contrast, claims 19-22 recite rapamycins and ascomycins produced by counter current separation. Citing the MPEP 2113 and *In re Thorpe*, 777 F.2d 695, 698, 227 USPQ 964,966 (Fed. Cir. 1985), the Examiner has asserted the proposition that patentability of a product does not depend on its method of production, but rather non-obvious differences in the product. Applicants respectfully submit that claims 19 to 22 are directed to products that are novel and non-obvious over Gletsos. In order to advance prosecution of this application however, and not in any way acquiescing to the position of the Examiner, claims 19-22 have been cancelled without

prejudice. Applicants reserve the right to file one or more divisional or continuation applications directed to the subject matter of the canceled claims.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent 5,616,595 to Chu et al. Since claims 19-22 have been canceled from the application, the rejection is now moot.

Claims 19-22 have also been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by EP 427680 A1 to Baumann. The rejection of claims 19-22 is moot since these claims have been canceled from the application.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent No. 5,665,772 to Cottens et al. As these claims have been cancelled from the application, the rejection is moot.

Claims 19 and 21 have been rejected under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claim 1 of U.S. Patent No. 6, 706,727. The rejection is now moot since claims 19 and 21 have been cancelled from the application.

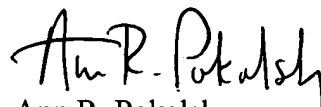
Applicants acknowledge the Examiner's finding that Claims 12, 13 and 17 contain allowable subject matter and would be allowed if rewritten to overcome the rejection(s) under 35 U.S.C. § 112, 2nd paragraph.

Accordingly, in view of the foregoing remarks and amendments, the present

application is believed to be in condition for allowance, which action is earnestly solicited.

Novartis
Corporate Intellectual Property
One Health Plaza, Building 430
East Hanover, NJ 07936-1080
(862) 778-7909

Respectfully submitted,


Ann R. Pokalsky
Attorney for Applicants
Reg. No. 34,697

Date: December 22, 2005

Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation

TAKAFUMI UCHIDA¹, FUMIHIRO FUJIMORI^{2,*} and NAOKI NAGATA^{3,*}

¹Institute of Aging, Development and Cancer, Tohoku University, Aoba, Sendai 980-8575;

²Department of Biological Science and Technology, Tokyo Science University, Noda; ³CREST, Japan

Received November 19, 2001; Accepted December 6, 2001

Abstract. Macrocyclic polyketides have generated great interest in biosynthetic chemistry because of the structural complexity and medicinal activities. The synthetic genes consist of the number and type of active sites of modular polyketide synthases. The cosmid library - prepared with the ascomycin (an antibiotics with immunosuppressive activity) - producer, *Streptomyces sp.* AA6554 genome was screened with an ascomycin ketosynthase gene probe, and one and a half modules were isolated. Database analysis shows that one of the modules consists of the genes coding a series of enzymes for the tetra-hydropyranose ring synthesis.

Introduction

Polyketides are natural products identified in various species and are especially abundant in fungi and actinomycetes. Genetic analysis of polyketide genes separated them into two classes (1). One of the class consists of macrolides which provide an excellent source to the pharmaceutical drugs. Ascomycin, C-21 ethyl analog of FK506 (2) is a 23-member macrolide (3). Ascomycin is also known as immunomycin and FK520. FK506 and rapamycin consisting of similar structures to that of ascomycin (Fig. 1) have potent immunosuppressive properties to inhibit T cell activation both *in vivo* and *in vitro* (3,4). These three compounds contain the pyranose-pipecolinyl region (C1 to C15; Fig. 1) which mimics leucine- (twisted amide) -proline peptide where peptidyl prolyl cis/trans isomerase (PPIase) binds to and causes various biological activities (5).

We identified the genes coding the synthetic enzymes for ascomycin tetra-hydroxypyranose ring, a part of pyranose-

pipecolinyl region where it binds to FK506-binding protein. The structure of the module of this gene is different from those of the FK506 synthase gene A, *fkBA* (6) and rapamycin synthase gene 3, *RAPS3* (7,8) which code the synthases for tetra-hydroxypyranose ring for FK506 or rapamycin respectively.

It is important to increase the genetic database of macrocyclic polyketide synthases. Such information will make it possible to manipulate the synthase genes, generate unnatural macrolides and increase the diversity of macrolides dramatically.

Materials and methods

Cloning. Genome DNA was isolated from *Streptomyces sp.* AA6554 and digested with *Sau3A* partially. The digested DNA was ligated into pWE15 cosmid with *Bam*HI sites at the ends (Stratagene). Ascomycin synthetic gene cluster was isolated by using the ketosynthase (KS) gene as a probe. The ascomycin KS gene was cloned by PCR. The primers for the PCR (5' primer, 5'-TTCGGGATCAGTCCTCG-3'; 3' primer, 5'-AGGATGACGTGGGCGTT-3') were designed to cover the highly conserved region of the KS gene by comparing the sequence of the KS gene for DEBS1 (2) and that for RAPS3 (7). The amplified product (1047 bp) was sequenced, compared with the other KS genes and confirmed to be a KS gene. The cosmid library was screened with the ascomycin KS gene as the probe. One of the positive clones was picked up, fragmented with sonication and subcloned into pUC19 plasmid to be sequenced.

Sequence analysis. The DNA sequencing was done on double-strand DNA templates with dideoxy method using an automatic sequencer (Applied Biosystems, Model 377 sequencer). The random sequences were compiled and the assembly was performed with the Applied Biosystem Auto Assembler (ABI). The deduced protein sequences were compared with sequences in the GenBank database using the BLAST program (9) and the alignments were performed using the PILEUP and CLASTW program (10).

Correspondence to: Dr Takafumi Uchida, Department of Pathology, Institute of Aging, Development and Cancer, Tohoku University, 4-1 Seiryō, Aoba, Sendai 980-8575, Japan
E-mail: uchidat@idac.tohoku.ac.jp

*Contributed equally

Key words: polyketide, ascomycin, tetra-hydropyranose ring

Results and Discussion

Streptomyces sp. AA6554, high producer of ascomycin was newly isolated from soil. We speculated that the biosynthetic

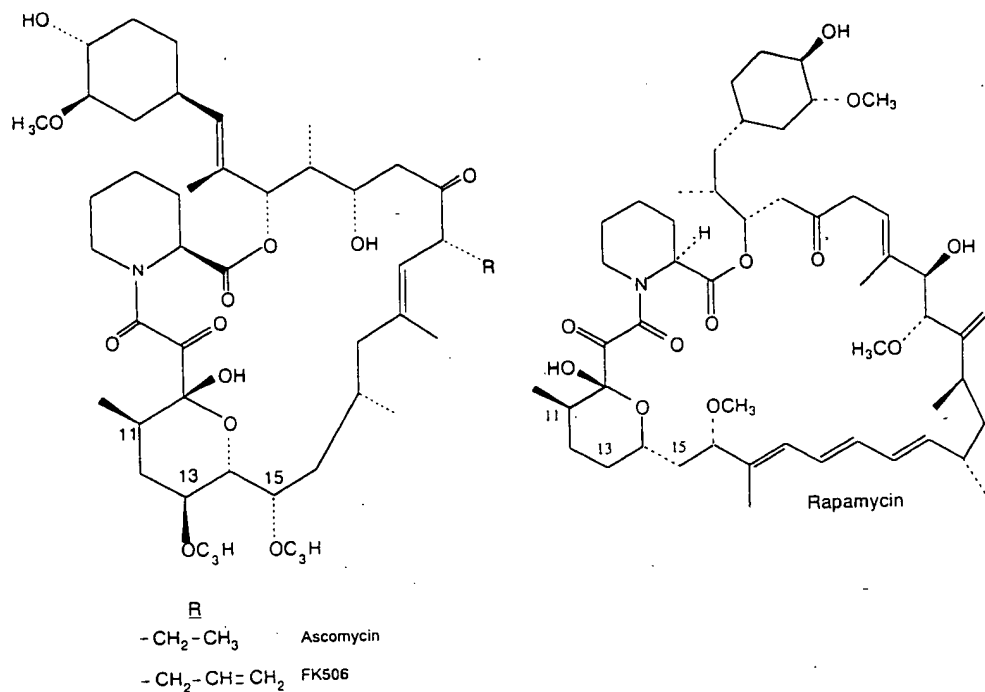


Figure 1. Structure of ascomycin, FK506 and rapamycin. Ascomycin, C-21 ethyl analog of FK506 is a 23-member macrocyclic lactone. Ascomycin is also known as immunomycin and FK520.

	ASC	FK506	RAPS	Consensus
ASC	REAGRLSEAS GRGSRANASA LRHEDRSQB PVAVVGNACR YPGGASPSBA LNRLLASGAE ANAEPPTDRG WDLGLFPHD PDRPGTSRAA EGGFLYDADR PDPEFGISF ASALVCPQD	..GTRAPVA AR.....TAA TAAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITHEPADRG WDVDACTYDPP PDALGHTFVR EGGFLDAGTQ PDAAEPGISF ASALAMOPQDB PLAIYGNACR LPGGVSSPBD LNRLLSGTD AVSGPTDRG WDVENLT...DHAGKSHRA EGGFLDAAG PDAGFPQISF ASALAMOPQDB P-A-VGNACR -PGG--SP-- LNRLL--SG-- A---PP-DRG WD---L---D-G---GGFL--A---PD---PFQISF ASAL--DPQD
ASC	RLLEBVAVPL LRAAGIDPVS LKGSSTGVTA GTALPGPTGT HIOKSAEGL YTGNAFSLVS GRVATTLGLE QPAVTVDTC SSSLVSHMLA CQALRQGEOT LALAGGVTVN AIPNVFTEB	RVLEBTSWRA FSAAGIIPDA ARGSDTGVPF CAFSIGTGTG ...ADTNGFG ATGSDTSVLVS GLSTPYGLE QPSVTVDTC SSSLVALRQA QSSLRSGECS DALVGVTVN ASPGGFVETB	RLVLEBTSWRA FSAAGIIPGS VAGSDTGVPF GATPGGTGIG ...ADLGGFG ATASRVSVLVS GRVSTPYGLE QPAVTVDTC SSSLVALRQA GTALRQGECS DALVGVTVN ATPGTVETB	R---LB--NB--B-AGI---GS-TGV--G---G-G---SVLS GR--T--GLE GT--TVDTAC SSSLV--R-A ---LR-GSC- LAL-GGVTVN A-P--P-BFS
ASC	RQGLAPDGR CKAFSADADG TAFSEGVGLV LRLRLSDARR HGRVIALIR GSAVNDQDAS NGLTAPNGFS QQRVILQALA HARLSPARVO AVEANGYCTH LGDFISVPGA ARHYGRDRPH	RQGLAPDGR ARATGACAGD TSFAGCAGAL VYRLSDARR HGRVIALIR GSAVNDQDAS NGLTAPNGFS QQRVILQALA HARLSPARVO AVEANGYCTH LGDFISVPGA ARHYGRDRPH	RQGLASDGR CKAFADAADG TQVAGGVGLV LRLRLSDARR HGRVIALIR GSAVNDQDAS NGLTAPNGFS QQRVIRAAALS NAGLSTARVO VVEANGYCTH LGDFISVPGA ARHYGRDRPH	RQ-GLA-DGR -KAF---ADG T---EG-G---RLSDA-R -GR-VLA--S GSA-N-DGAS NGL-APNGFS Q-RV---AL- NA-L--A-VD -VANGYCTH LGDFIS---TG-DR--
ASC	DRPLVLCVSK SHIGHTQGAQ GVAGVKNIN AMRIATLPAT LHVKPTPBV SMGTGAVRLT TBAGVMPRGE RPRAAVVSF QISGTHARLI LEQAPADBPV APDRTGPTA VDIAPPLDTA	ATPLLLGSLK SHIGHAQAAS GVAGIKNVQ AIBGRLPPT LHVDPSPBV SMGTGAVRLT TBAGVMPRGE RPRAAVVSF QISGTHARLI LEQAPADBPV APDRTGPTA VDIAPPLDTA	DQPLVLCVSK SHIGHTQGAQ GVAGVKNIN ALQRLPPT LHVDPSPBV SMGTGAVRLT TBAGVMPRGE RPRAAVVSF QISGTHARLI LEQAPADBPV APDRTGPTA VDIAPPLDTA	---GS-K SHIGHTQ-A- GV-G-IRM- A-----P-T LN---P-EV -W-CAY-L- T---WP---R-RRA-VSSF G-SGTNAR--LI-----P-----
ASC	PPIDTAEPVG .GRUVVPMVS ARTQALQDD ABALAAHLTA HPLPTTGVG WSLARTSAP ENRAVAIGDS HOBLLAAVRA LADGSHFGL TATTAARSG GTALNTTGG SQRPGTGRGL	GPFAAPFSA PGSLPLVLS ARSPALDQ IGRALATDT GPGVDRAVA QTLARLIEP TRAVILGDTV ACAPADQAD ELVVFVSCQG TQKFMGBQLFLVIS ARTQSALTIE IGRALATLAA SPGVDRARA SILANTSEVP ENRAVILGDDV TGTGTAVSDP RAVVVFVSCQG SQRAGMGBLP-S AR---L---L---L---PG---V---P---RAV--GD-----GQC-----G--L
ASC	TDATPVYARA PDVACALDIT BLRPLGLIA PGDNFBLOR TATAQPALTA VHVACFALVB STGLTPTSLT GHSVGLVAA RVAGVLSLPP ACALVAABCR LQALPAGGA HAAVATREIQ	AAAFVFPADA MDAIRLID...DP.....D.P...ND PIRSQHTLTA HQAATALLR SWDITPBAVI GHSGLBITAA TAAGILSLDD ACTILITBAR LNTLTPPGA NVTVLTSERB	AAAFVFPARI HQVHOLLID...VF.....D...LEVMS TGAQPALTA HQVAFGLLE SMQVPSLDD ACTILVBARA LQALPAGGV HAAVATREIQ	-A-PV-A---LD---P---D---Q-LFA---A--L--S---GHS-GE-AA---G--SL-D AC-----R LN--L--N---N-----A--
ASC	VVPLLAGASD EVLLAAVNGP TIVUVSGAAB TVORIAARTK ERCHRTKRL VSHAPSPLL DPLDOPROV ARRLTSPFR IPVISCVTGR QALPQLRDP LYNVRVVRP VPHDGLRLT	AQALAPFG...VIAAVNGP HSVVLSGDD AVLDVAQRLG ...IHURLP APHAGHSAH BPVAAELAT TRELAYDRPB TAIPH.....DP...T.TA SYWAGVVRP VLTHA....GRFV	AVAVLGG...VIAAVNGP HSVVLSGDDA AVLDVAQRLG ...KWTBLA TSHAPSPAXH SNQVPSLDD ACTILVBARA LQALPAGGV HAAVATREIQ	---L---V-A-AV-GP -SVV-SG---A---RL---RA-HS---L---P---GHS-GE-AA---G--SL-D AC-----R LN--L--N---N-----A--
ASC	EGEGVITRLE LGPDVLTEN AQDALATGAP GAQDTSAPV FATALRPGRD SPRTLLTALA LTHIGATVD FAALP..BD AGAYDLPTTR PQRQREVP APNATADVRA VGLTGTDRPL	EPDPAVFBVI G.....P.G.QQLSPVLD GIALQNGTAD VPHALSTALA RLPTGATLD MSRLGG.AS RSDPQVPSVA PQRPTVRES APPATADS.....GRFVU..VA STDAVVEL G.....ADRLCARLVD GYANLEG.DE EIQALICALA LNVHNGVTVD MPALGDAPA TAVLDPTTA PQRQREVP TQATAGG.....NPLDAV.....E---LA---G--O-----P---PQ--RIN---ATA---NF--
ASC	LQAVVFEDC GILITGLSP YTHSLADEZ IADSVPLFGT ALLELYLLAA ARTORBELVD LTLSTPLIL EGGAVHQLA VTAPDSTGR TWTIASPAD QGPDAAAR HBAATGLADA	LGTGVAVAGS...PGRVPT GPVACADRA.....AV FIABALALAA DATOCATVEG LDVTSVPPGS ABGRATAQIM VDBPADGRB RPTVTRVG.DAP.....NT LBAAGVLRPG	LGSVAVLAA...SOVLEP AVSRSGDLM LROQTLPAT VPHALSTALA DEACGLVGG LNVHALLLP DDGAVQVQM VSEPDAGRN RESIRARTSD SEP.....NT RLATATLATR	L---V-----D-----E--L-AA-----L-----V-----R-----

Figure 2. Continued on the next page.

ASC	ATTPFAAAPP	TIAPWPPAGAV	PVDVADLIER	LTAQGTATGP	AFRGLBTANR	LGBNPAFVR	LAPERRGAD	ATGVNPFALLO	SALBPVBEFL	HGGFVDPGFE	ATVLPFSPG	GVLNFTPGAT
fkA	RVFQF...AV	DIAPWPPAGAV	PA...D...	...GLPQANR	AAQGVVPAAS	VOSP...D	GFVANFDLLO	AVFS...	...VGDG.S	...RQPTGHR	DLAVBASDAT	AVGSG
RAPS	GTVSG...VQA	GSAPWPPAGAV	PVETGVF...	...SLQGVVR	RQHEVVPABVA	LOST...HAT	TIALRFPALLT	AALT...	...TAG...	...SETFAASQ	ALTLNHRPA	
Consensus	-----	---ANPP-GAV	P-----	---L---VR	-----	-----	---RP-LI-	-----	-----	-----G-	-----	
ASC	RLRVRTTPIA	PDVTIRITD	ONGAPVATID	SLGLREVPAD	ANRVSNSATA	DAFLTELQNG	PYPVSASAV	FTANRVLVGA	RBPDLGLPAN	FDLAALRAAL	DDGEVPVDDV	VLACPGAPDT
fkA	VLKACLTARD	SGVVELAAND	GAGMPVLTA8	SVTLGDEVASA	GG...SDRS	DG...LRLRHL	P...VAREAT	GGADSLPQCY	T...LITATN	P...DD...	...PDD	
RAPS	SLRVRLISDD	DGTLSDVADT	SYGLPVLTVR	SLTLRTVPVT	SP...ATS	TDGLTLTMA	EIFAPQSTGL	TVGRFEDLVS	D...ADVPVPEV	AVFT...	...ALPOS	
Consensus	---LF-----	---D-----	---PV---	S---L---V---	-----	---L---L---	-----	-----	-----	-----	---PU-	
ASC	SADPDQTPAR	VRAAAYPVLG	ALRTNLTQDR	PSGARLYIAT	RGAVATGPGD	APADLATAPV	NGLVRAAQA8	RFQRMVLLDL	DDDTASRDAL	RTAFPAANAD	ABSELAVRAG	TABVPLVR:
fkA	PTNPHTPTFR	THQTTEVLT	ALQHLITTM	RT...LIVNT	...TTPPPG	...AAV	TGLTRTAQNE	RFGRIDLIET	HEHTP...	...LPLTQLTL	NQPLRLTMM	TLBTPBLTVI
RAPS	SENF...LEQ	P...RAVMDPQ	AVQTLGGGR	PTOSTLVVPT	G...TLAAAG	...V	SLKRSASQE	EPGRFVLVS	DDTLAP...	...DQLAATVGL	DFPLRVSGD	VLDGTVASRL
Consensus	-----	---VL-	A-----	---L---T	-----	---V	---GL---AQ-	EP-R-----	-----	-----	---L---	---
ER												
ASC	RPDRD...T	P...ARALDPDG	TALITGGTGA	LORLVARNLV	TANGVRELL	VSRFGGITE	IDAFADBLTS	LGAADVRYTA	CDAADPEALA	VLLATLPEAR	PLTAVVHAAG	VDDGVVTSN
fkA	TYHNTITTTT	PHVPLNPNH	AILITGGSGT	LAQILARHLN	SPH...TTL	LSRTF...PP	...FIT	PGTHIP...	CDTDPPTQIT	QALTRIPQ...	PLTGTETAA	TIDDTATML
RAPS	NASGS...B	P...RAVMDPQ	TVLITGGSGV	LQIAARHLV	ARGVARNLL	LSRSAPRDL	IN...GLG8	LGAHVET.AA	CYSDRAALA	QVLAGVSP8	PLTAVVHAAG	VLDGTVASRL
Consensus	-----	P-----	---LITGG-G-	L---ARHL-	---L---	---SR-	-----	-----	---D---	---L---	PLT---B-A-	---LDD-----
ASC	TPQQLDTVLA	FELDAAPBLE	RLTRTKDPAA	PVMFSSAAAT	NGMGQAMTA	AANMFLWALA	ERRRANGRAS	HALAWGLLAS	AGCHTGLDLO	ADLARNHARS	IAPVSHIQAAL	TLLDTALTTO
fkA	TPQQLDTVLA	PRADAAPBLE	HTQHLITTM	PVLTSAAAT	LGSPPQAMTA	AANAPDLALA	ERRRANGRA8	TTIANGHMBT	TYLTSQSLTD	SORDIRAGG	PLTGTETAA	RLTDAVSG8
RAPS	TAQQLDTVLA	PRADAAPBLE	HTQHLITTM	PVLTSAAAT	LGSPPQAMTA	AANAPDLALA	ERRRANGRA8	TTIANGHMBT	TYLTSQSLTD	SORDIRAGG	PLTGTETAA	RLTDAVSG8
Consensus	T---L-T-L-	PK-D-AWEH	---T-----	PV--SSAA--	-G-CQ-NYA	AA--PL-ALA	-----	---G---	---G---	---T---L-D	---R-G	---D---
ACP												
ASC	HATLVPAFID	LAALTRAAAT	GFLPFLRL	VHVPTAPRYT	TGANSLSBL	AGLPAGEGRD	LVBLVRDQV	ATVLAHFAPE	AIBPKAFQD	LCPDLSLTD	LRNRLAATG	IRIPATVIFD
fkA	EDVLAANKAD	PAQ...NA	GDVPLILSG	RKSARKTART	G...QTAPQL	ALPAAADRT	ALVTLVEDAT	AAVGLHADAS	GIAPTTTFPD	LQDLSLTA8	LRNRLAATG	IRIPATVIFD
RAPS	NPILVAAPND	PVMD...A	EVPALLRSLH	RPVARRAAT	S...DSSARVL	AAAPADPKED	ALLVLRDSDA	ALVGLHADAS	TIPAAAPFD	LQDLSLTA8	LRNRLAATG	IRIPATVIFD
Consensus	---A-D	-----	---R---T	---	---L---	---	---LV-D-	A-VL-H---	-----	---D---	LRN-L-L-STG	---R---TD
KS												
ASC	TFTDQALVGT	LRBLTGAPA	AAPLFTATA	AAADDPFIV	VGNACRYPGG	AGSPBLFRL	VADGVDAIGE	PPDGRNDLA	GLFDFPDHDT	GTSIARBGOT	LISAPFZDAS	PPGISPRBAL
fkA	ETPFRVLAAS	LRTDLGCT	AAAPL.ARTA	RTNDEPLAV	VGNACRLPGG	VSPDRLVRL	VASGTDALTE	PPDGRNDID	RPDPPDPADP	GTITVRHGOT	LSBAAGFZDA	PPGISPRBAL
RAPS	TFTDQALVGT	LRTDLGCT	AAAPL.ARTA	RTNDEPLAV	VGNACRLPGG	VSPDRLVRL	VASGTDALTE	PPDGRNDID	RPDPPDPADP	GTITVRHGOT	LSBAAGFZDA	PPGISPRBAL
Consensus	---TF---L---	L---G---	---AP---	---D---	VGNACR-PGG	---SP8-LVRL	V--G-DAL-	PP-DGND-	---PDPPD-	G---T---GDP	L---A---FZDA	PPGISPRBAL
ASC	ATDPQQLKLL	ETAWAPBSA	GIDPVSLEGS	KSAVITGVNT	DTQGRFLGR	TPRGVGRHLM	TGSTFISASG	RYAFTYGLRG	PAVTVDTACS	SSLVAMHLLA	QALRQBCLE	ALAGGVTVMA
fkA	ANDPQQLKLL	ETAWAPBSA	GIDPVSLEGS	DTQGRFLGR	DTQGRFLGR	AGDILGGTGA	TATQNSVLGS	RLSTYFGHMG	PAVTVDTACS	SSLVAMHLLA	QALRQBCLE	ALAGGVTVMA
RAPS	ANDPQQLKLL	ETAWAPBSA	GIDPVSLEGS	DTQGRFLGR	DTQGRFLGR	AGDILGGTGA	TATQNSVLGS	RLSTYFGHMG	PAVTVDTACS	SSLVAMHLLA	QALRQBCLE	ALAGGVTVMA
Consensus	A-DPQQR---	E-W--FF-A	G-I-P--RG-	---G---	---TG---	---G---	T---SSG8	R---FG-EG	PA-T-DTACS	SS-VA-E-A-	---LR-QBC-L	AL-GGVTVMA-
ASC	TPNTFVPSR	QRLGADGRC	KFFAAAADGT	QWGGIGLVL	LSRLSDARRN	GRVLAIVRG	SAVMDQDASN	GLTAFUGPSQ	QRVIRQALAN	AKLSPASVDA	VRABGTOTTL	GDPIBAQALL
fkA	TPNTFVPSR	QRLGADGRC	KFFAAAADGT	QWGGIGLVL	LSRLSDARRN	GRVLAIVRG	SAVMDQDASN	GLTAFUGPSQ	QRVIRQALAN	AKLSPASVDA	VRABGTOTTL	GDPIBAQALL
RAPS	TPNTFVPSR	QRLGADGRC	KFFAAAADGT	QWGGIGLVL	LSRLSDARRN	GRVLAIVRG	SAVMDQDASN	GLTAFUGPSQ	QRVIRQALAN	AKLSPASVDA	VRABGTOTTL	GDPIBAQALL
Consensus	TP---VEP-R	QRLGADGRC	KAF---ADGT	---BG-G-L-	---SLSDA---	G---VLA--R-	SAVMDQDASN	G--AFNDPSQ	QRVIT--AL--	A-L---VD-	VRABGTOTTL	GDPIBAQALL
ASC	ATTGRBEPED	BPLMLQSIKS	NICHTQAAAG	VAGVIKMKVA	HREGLLPASL	HIDBFSQEV8	NODGQVRLT	BAVENPFRAR	PRRAVSSYG	ISGTNANVIL	EQAPORAFDT	GRPKFPDDGP
fkA	ATTGQDR...D	TYLIGSVAS	NICHTQITAG	LAGVIKMKVA	HREGLLPASL	HIDBFSQEV8	NODGQVRLT	BAVENPFRAR	PRRAVSSYG	ISGTNANVIL	EQAPORAFDT	GRPKFPDDGP
RAPS	ATTGQDR...E	TYLIGSVAS	NICHTQITAG	LAGVIKMKVA	HREGLLPASL	HIDBFSQEV8	NODGQVRLT	BAVENPFRAR	PRRAVSSYG	ISGTNANVIL	EQAPORAFDT	GRPKFPDDGP
Consensus	ATTG--B---	---L-LGS--S	NIGH-Q--G	---GVIKMKVA	---B---F-L-	R-D8FS-HV-	R--G-V-L-	S---RP--R	PRRA-VSS-G	---SOTNANVIL	E-----	---
AT												
ASC	E...VVPVLS	ARGATALRQD	APALVARIAT	GPLASSABVC	YSLIKSRTLF	DERAVVVGED	EALTAALBA	LAAGSHPVVC	VGPQAVVSGG	GVPQVLPVFG	QCSQWVGGA	GLDASPVVFA
fkA	...LVPLPVS	ARTESSALQ	YHRLGIVRG	...ARLAAVA	DGLVGRGVTF	CHRAVLLGDS	...TVAG...	VAGARRS...	...T...	...VEVFP	QCSQWVGGA	GLDASPVVFA
RAPS	ASDVPFLVIS	ARTSSALQ	YHRLGIVRG	...ARLAAVA	DGLVGRGVTF	CHRAVLLGDS	...TVAG...	VAGARRS...	...T...	...VEVFP	QCSQWVGGA	GLDASPVVFA
Consensus	---P-S-A	---L---	---	---	---	---RAV--G8	---TVAG...	---A---	---	---V-VFP	QCSQWVGGA	GLDASPVVFA
ASC	ARVACB8RAL	APVGV8LTD	VLRGV8DAGD	LGRV8V8VVP	LWAVW8SLAA	WVAV8CVNPA	AVV8V8V8V8	AAACVACALT	LEDGARVVAL	88PALR.8LA	GGGANASIAL	GC88V8GL8S
fkA	ARVACB8RAL	APVGV8LTD	VLRGV8DAGD	LGRV8V8VVP	LWAVW8SLAA	WVAV8CVNPA	AVV8V8V8V8	AAACVACALT	LEDGARVVAL	88PALR.8LA	GGGANASIAL	GC88V8GL8S
RAPS	ARVACB8RAL	APVGV8LTD	VLRGV8DAGD	LGRV8V8VVP	LWAVW8SLAA	WVAV8CVNPA	AVV8V8V8V8	AAACVACALT	LEDGARVVAL	88PALR.8LA	GGGANASIAL	GC88V8GL8S
Consensus	---R-EC---L	---N-L-	VL---	---RV-V-QP-	---RA-VSLAA	---GV-F-	AV-Q8V8V8	AAACVACALT	LEDGARVVAL	88PALR.8LA	GGGANASIAL	GC88V8GL8S
ASC	GLGDRVAAVY	VAAVNGPAST	VYSGPF88QA	AAVAC88C8T	8888888888	AS88888888	AG88888888	VE88888888	V88888888	V88888888	V88888888	8888888888
fkA	G...V...VY	VAAVNGPAST	VYSGPF88QA	AAVAC88C8T	8888888888	AS88888888	AG88888888	VE88888888	V88888888	V88888888	V88888888	8888888888
RAPS	G...V...VY	VAAVNGPAST	VYSGPF88QA	AAVAC88C8T	8888888888	AS88888888	AG88888888	VE88888888	V88888888	V88888888	V88888888	8888888888
Consensus	G-----	---AL-NGP-ST	V--G-F--V-	-----	---R-L-VDT	ASH---V-I	---	---	V---TV-G-	V---V---	V---V---	---A---L-
ASC	GAGRVVFI8V	ST88V8LTH8	QIT888888	ALT888888	H88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888
fkA	GS...LPI8C	SA88V8LTH8	QIT888888	ALT888888	H88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888
RAPS	GS...LPI8C	SA88V8LTH8	QIT888888	ALT888888	H88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888
Consensus	---G-S---	---PVL---	---	---TV---L-D-	---G---	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888	LA88888888
ASC	DG88888888	L88888888	Q88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888
fkA	DG88888888	L88888888	Q88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888
RAPS	DG88888888	L88888888	Q88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888	V88888888
Consensus	---RA-DE--C-	---BL-----	P---	L--P-TQ-V-	---V-V---	---G-R-V-V---	---	---	---R-V-V---	---	---	---
ASC	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888
fkA	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888
RAPS	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888
Consensus	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888	QWPPQ88888

Figure 2. Comparison of the deduced amino acid sequence of ascomycin synthase gene with those of *fkA* and *RAPS3*. The consensus sequence is shown under their sequences. One complete module containing a KS, an acyltransferase (AT), a dehydratase (DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified.

genes for ascomycin consist of a complex of modules like other macrolides, such as erythromycin (2,6), rapamycin (7,8) and FK506 (11). The sequence of β -ketoacyl synthase

(KS) gene of ascomycin synthetic genes is similar to those of the other macrolide synthetic genes. The amino acid sequence of KS of 6-deoxyerythronolide B synthase (DEBS) (2) and that

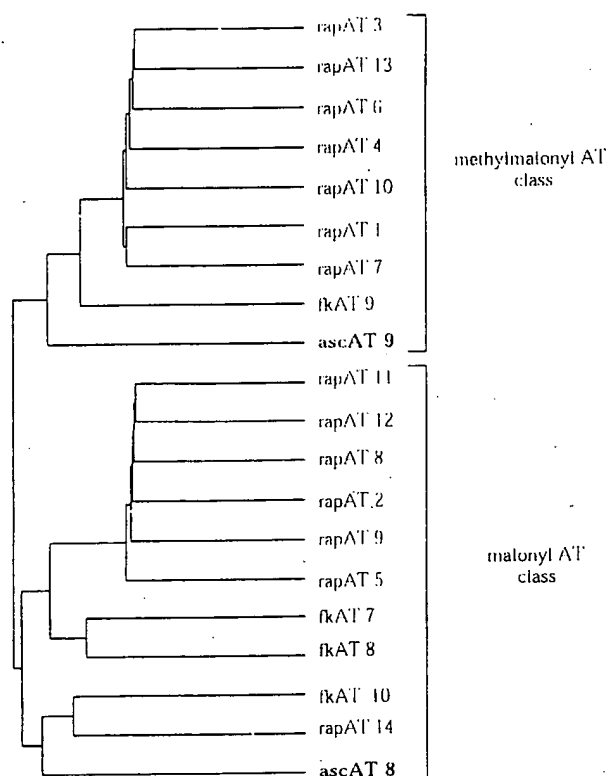


Figure 3. The PILEUP analysis of acyltransferase (AT) domains in these modules. The analysis suggested that the substrates for the identified ATs are acetate (ascAT8) and propionate (ascAT9) respectively.

of rapamycin synthase (RAPS) (7,8) showed high homology to each other, but they have little homology to those for aromatic polyketide synthetic genes (1). The DEBS KS gene probe hybridized to RAPS KS gene or vice versa but neither KS gene probe hybridized to the KS genes of aromatic polyketide synthases (data not shown). We compared the sequences of DEBS1 and RAPS3, and synthesized the PCR primers covering the high homologous sequences around the active sites of the KS genes. DNA fragment (1.1 kb) containing KS active sites was amplified with PCR using the primers. The sequence of the fragment showed high homology to those of DEBS1 and RAPS3 KS genes, especially the active site regions of the KS genes are very well conserved. These results showed that the isolated fragment is an ascomycin KS gene, so we used it as the probe to screen the ascomycin synthase genes.

Fifty-four positive clones were isolated from the cosmid library. Southern-blot analysis of genomic DNA from ascomycin producer cells suggested that the total size of the ascomycin synthase genes is included in 82 kb. We chose the number 44 clone carrying 8 kb insert and determined the sequence completely. Comparison of the deduced amino acid sequence of the clone with the proteins in the database revealed that it contained the consensus active sites of fatty acid synthases and polyketide synthases (12). One complete module containing a KS, an acyltransferase (AT), a dehydratase

(DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified (Fig. 2). The amino acid sequence of these enzyme domains corresponded to the module 12 and 13 of RAPS3 and module 8 and 9 of fkbA.

The sequence of KS domain was conserved well between the macrolide synthases. But other enzymes, AT, DH, ER and ACP showed less homology to each other. The KR domain contains a potential NAD(P)H binding motif, GXGXXAXX-XA (8,12). The KR domains of the modules indicated that the KR is active because it contains LGDSL motif where 4'-phosphopantetheine attaches (12). The PILEUP analysis of AT domains of these modules showed that the substrates are acetate and propionate respectively (Fig. 3). The main structure of ascomycin is speculated to be synthesized with poly-merization of acetate and propionate in the following order; shikimic acid - propionate - propionate - acetate - butyrate - propionate - acetate - acetate - propionate - acetate - pipecolic acid. This sequential arrangement exists only at the C10 to C13 position of ascomycin, which gives a pyranose-ring, in other words tetrahydropyran (Fig. 1). Taken together, we concluded that these modules correspond to modules 8 and 9. DH was identified in the module 8 (Fig. 2), although DH activity in this module is not required for ascomycin biosynthesis. The motif, HxxxGxxxxP is speculated essential for DH activity. The motif of this DH has the mutation of the Gly to Asp (12). DHs are sometimes inactivated by mutation or deletion of amino acids at the active sites, for example the DH of fkbA module 8 and RAPS module 2, 5, 11 and 12 (6-8) contain a five amino acid deletion in the active sites. So the mutated DH in this module is probably an inactive one.

In conclusion, we cloned a part of ascomycin synthetic genes, which code the enzymes for the ascomycin tetra-hydropyranose ring formation.

Acknowledgments

We thank C. Miyamoto, T. Okuda, Y. Anzai and T. Minamitake for their help through this project.

References

1. Hopwood DA and Sherman DH: Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu Rev Genet* 24: 37-66, 1990.
2. Cortes J, Haydock SF, Roberts GA, Beviit DJ and Leadlay PF: An unusually large multifunctional polyketide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. *Nature* 348: 176-178, 1990.
3. Hatanaka H, Kino T, Miyata S, Inamura N, Kuroda A, Goto T, Tanaka H and Okuhara M: FR65814, a novel immunosuppressant isolated from a *Streptomyces*. II Fermentation, isolation and physico-chemical and biological characteristics. *J Antibiot* 41: 1592-1601, 1998.
4. Kino T, Hirayama N, Miyata S, Inamura N, Nishiyama N, Yajima T, Goto T, Okuhara M, Kohsaka M, Aoki H and Ochiai T: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. *J Antibiot* 40: 1256-1265, 1987.
5. Rosen MK, Standaeft RF, Galat A, Nakatsuka M and Schreiber S: Inhibition of FKBP rotamase activity by immunosuppressant FK506: twisted amide surrogate. *Science* 248: 863-866, 1990.
6. Motamedi H, Cai SJ, Shafice A and Elliston KO: Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506. *Eur J Biochem* 244: 74-80, 1997.

7. Schwecke T, Aparicio J, Molnar I, Konig A, Khaw LE, Haydock SF, Olijnyk M, Caffrey P, Cortes J, Lester JB, Boehm G, Staunton J and Leadlay PF: The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. *Proc Natl Acad Sci USA* 92: 7839-7843, 1995.
8. Aparicio JF, Molnar I, Schwecke T, Konig A, Haydock SF, Khaw LE, Staunton J and Leadlay PF: Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hydropiscus*: analysis of the enzymatic domains in the modular polyketide synthase. *Gene* 169: 9-16, 1996.
9. Altschul SF, Gish W, Miller W, Myers E and Lipman DJ: Basic local alignment search tool. *J Mol Biol* 215: 403-410, 1991.
10. Devereux J, Haeberli P and Smithies O: A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12: 387-395, 1995.
11. Bevirt DJ, Cortes J, Haydock SF and Leadlay PF: 6-Deoxyerythronolide-B synthase 2 from *Saccharopolyspora erythraea*. Cloning of the structural gene, sequence analysis and inferred domain structure of the multifunctional enzyme. *Eur J Biochem* 240: 39-49, 1992.
12. Donadio S and Katz L: Organization of the enzymatic domains in the multifunctional polyketide synthase involved in erythromycin formation in *Saccharopolyspora erythraea*. *Gene* 111: 51-60, 1992.